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Technological and safety assessment of selected lactic acid bacteria for cheese starter cultures design: Enzymatic and antimicrobial activity, antibiotic resistance and biogenic amine production

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ABSTRACT

Twenty lactic acid bacteria (LAB) isolated from artisanal cheeses and previously selected for their technological properties were screened for their enzymatic activities, antimicrobial activity and safety. The aim was to select those LAB strains that were safe and showed advantageous properties for the development of starter cultures for cheese making, discarding those that could transfer antibiotic resistance or produce any toxic biogenic amines. Aminopeptidase activities were detected in most strains, particularly high for the substrate leucine arylamidase, and most lactobacilli and *Leuconostoc* strains showed high β-galactosidase activity. Glutamate dehydrogenase (GDH) activity was detected in 13 strains, although the activity values varied widely. All strains showed antimicrobial activity against the indicator microorganisms due to acid production. However, only one of the *Lactiplantibacillus plantarum* strains showed an inhibitory activity against *Enterococcus faecalis* due to bacteriocin-like compounds. In particular, *Levilactobacillus brevis* TAUL1567 and *Lactiplantibacillus paraplantarum* TAUL1399 showed resistance to tetracycline and ampicillin, respectively, above the cut-off values and were therefore excluded. Tyramine was only produced by *L. brevis* TAUL1567 (193.15 μg ml^{-1}), while putrescine was produced by this strain and two strains of *Lactococcus lactis*. Finally, 14 strains produced γ-aminobutyric acid (GABA), five of them at concentrations around or above 100 μg ml^{-1}.

1. Introduction

Lactic acid bacteria (LAB) are used as starter cultures in the production of fermented foods and are therefore of great economic importance as they determine the organoleptic, rheological and nutritional properties of fermented foods. In cheese production, LAB play an important role in the biochemical events that take place during ripening, such as proteolysis and lipolysis, which contribute significantly to the development of flavour and aroma. Therefore, the technological characterisation of LAB strains through the study of their enzymatic activities is crucial for the selection of specific strains to be used as starter cultures in cheese production (Câmara et al., 2019; Redruello et al., 2021; Yogeswara et al., 2020). In this context, glutamate dehydrogenase (GDH) is a key enzyme in the conversion of amino acids produced during proteolysis (Tanous et al., 2002). Transamination is the central reaction in amino acid catabolism in LAB, leading to the formation of aromatic compounds. During amino acid transamination, aminotransferases transfer the amino group of an amino acid to an α-ketoacid, and subsequently the resulting α-ketoacids are degraded by additional reactions to the various aromatic compounds, which are aldehydes, alcohols, carboxylic acids and sulfur compounds (Pudlik & Lolkema, 2013). In general, amino acid conversion by LAB is limited by their low production of α-ketoglutarate, as α-ketoacid is essential for the first step of conversion. For this reason, LAB strains with high GDH activity, which is able to catalyse the deamination of glutamate, present in large amounts in cheese, into α-ketoglutarate, could accelerate, intensify or diversify flavour formation in cheese (Mazhar et al., 2020; Tanous et al., 2002).

In addition, there are other aspects of LAB strain selection that may be of interest from the point of view of starter culture design. One of these is the antimicrobial activity of LAB strains (Mani-López et al.,

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2021). During LAB's growth, compounds derived from their metabolism can be produced that have antimicrobial activity (Agostini et al., 2018). The main antimicrobial activity of LAB is due to their production of organic acids that prevent the growth of pathogenic or spoilage microorganisms (Favaro et al., 2015), but some of them can also produce bacteriocins or bacteriocin-like compounds that specifically inhibit the growth of certain microorganisms (Silva et al., 2018). Another interesting feature of LAB strains is their ability to produce compounds that promote beneficial health effects, such as γ-aminobutyric acid (GABA). GABA is a non-protein amino acid produced by the decarboxylation of L-glutamate or its salts by the enzyme glutamate decarboxylase (GAD) (Falah et al., 2021), whose production increases as a physiological response to acidic conditions (Luo et al., 2020). Due to its health benefits (Jitpakdee et al., 2021; Kanklai et al., 2021), the food industry is interested in producing GABA-enriched foods, and cheese may be a good candidate due to the natural ability of some LAB strains to produce GABA (Valenzuela et al., 2019).

LAB strains isolated from traditional fermented products and selected for their technological properties represent a very interesting niche for the development of new starter cultures. However, it is essential that these strains are safe for this use. Although the European Food Safety Authority (EFSA) has granted Qualified Presumption of Safety (QPS) status to most LAB species and the US Food and Drug Administration (FDA) considers them Generally Recognised as Safe (GRAS) (Kanklai et al., 2021), there are not many studies on the safety of LAB compared to other bacterial groups (Chen et al., 2019). Some strains from the microbiota of traditional fermented dairy products may carry antibiotic resistance genes that can be transferred to pathogenic bacteria through the food chain, leading to the spread of antibiotic resistance (Sirichoat et al., 2020; Stefańska et al., 2021). On the other hand, some LAB strains can produce toxic biogenic amines (BA) such as histamine, tyramine or putrescine, which can accumulate in food and cause food poisoning (Ladero et al., 2011); Ma et al., 2020; Moniente et al., 2021). Therefore, the absence of antibiotic resistance and the search for non-BA producing LAB strains are criteria for the selection of LAB strains for use in the food industry (Durak-Dados et al., 2020).

Taking into account all these aspects that are crucial for the use of LAB strains in the design of starter cultures, the aim of this work was to evaluate the technological and safety properties of previously selected wild LAB strains prior to the design of starter cultures. Desirable enzymatic activities, antimicrobial activity, antibiotic resistance/susceptibility status, production of toxic BAs and GABA production capacity were evaluated to exclude strains that might not be suitable for use as starter cultures.

2. Materials and methods

2.1. Strains, media and culture conditions

Table 1 lists the bacterial strains used in this study (LAB and control strains). *Levilactobacillus*, *Lactiplantibacillus*, *Lacticaseibacillus* and *Leuconostoc* strains were grown in MRS broth (Oxoid, Basingstoke, UK) at 32 °C; while *Lactococcus* strains were grown in M17 broth (Difco, Becton Dickinson, Sparks, MD, USA) supplemented with 1% (w/v) glucose (M17-Glu) at 32 °C. *Enterococcus faecalis* was grown in MRS broth at 37 °C. *Listeria monocytogenes* was grown in tryptone soy broth (TSB, Oxoid) at 30 °C. *Staphylococcus aureus*, *Salmonella enterica* and *Escherichia coli* were cultured in TSB at 37 °C. *Clostridium tyrobutyricum* was grown in Bryant-Burkey broth (BBB, Condalab, Madrid, Spain) in anaerobiosis at 37 °C. Anaerobic conditions were achieved by using the Anaerocult A (Merck, Darmstadt, Germany) in anaerobic flasks. For solid formulations, bacteriological agar (VWR International, Geldenaaksebaan, Belgium) was added to the liquid media.

Table 1
Species, strains and relevant features of the microorganisms used in this study.

Species and strains	Features studied
Lactococcus lactis subsp. *lactis* GE44[a], TAUL88[b], TAUL227, TAUL266, TAUL8000, TAUL9000	EP[c] GABA[d] AM[e]
Leuconostoc mesenteroides subsp. *dextranicum* TAUL1342	BA[f] AR[g]
Lactiplantibacillus paraplantarum TAUL1365, TAUL1399, TAUL1453	
Lactiplantibacillus plantarum subsp. *plantarum* TAUL67, TAUL180, TAUL188, TAUL1368, TAUL1569, TAUL1641, TAUL1667, TAUL1692, TAUL1694, TAUL1700, TAUL1765	
Levilactobacillus brevis TAUL1567	
Lacticaseibacillus paracasei TAUL1508, TAUL1752	
Listeria monocytogenes CTCE4031[m]	Reference strain for AM [h]
Staphylococcus aureus CTCE240[m]	
Enterococcus faecalis CTCE481[m]	
Escherichia coli CTCE101[m]	
Salmonella enterica CTCE4594[m]	
Clostridium tyrobutyricum CTCE4011[m]	
Enterococcus faecalis V583 [i]	Control for BA
Lentilactobacillus parabuchneri IPLA11150 [j]	
Furfurilactobacillus rossiae D87 [k]	
Lactococcus lactis LEY6 [l]	

[a] GE: strains of Genestoso cheese (González et al., 2010).
[b] TAUL: strains of the "Tecnología de los Alimentos – Universidad de León" collection (Abarquero et al., 2022).
[c] EP: enzymatic profile.
[d] GABA: γ-aminobutyric acid production.
[e] AM: antimicrobial activity.
[f] BA: biogenic amines production.
[g] AR: antibiotic susceptibility.
[h] Reference strain for AM: this work.
[i] *Enterococcus faecalis* V583: reference strain for tyramine production (Fernández et al., 2004).
[j] *Lentilactobacillus parabuchneri* IPLA11150: reference strain for histamine production (Coton et al., 2010).
[k] *Furfurilactobacillus rossiae* D87: Reference strain for putrescine production (Coton et al., 2010).
[l] *Lactococcus lactis* LEY6: reference strain for putrescine production (Ladero et al., 2011).
[m] Type strain of Spanish Type Culture Collection (CTCE), Spain.

2.2. Lactic acid bacteria: molecular identification and PCR fingerprinting

Twenty-four LAB strains isolated from traditional cheeses and selected for their desirable technological properties (Abarquero et al., 2022; González et al., 2010) were included in this work to exclude isolates belonging to the same strain (Table 1).

Molecular identification was performed by amplification and sequencing of the 16S rRNA gene. Total genomic DNA was purified from overnight cultures using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, MO., USA) according to the manufacturer's recommendations. Purified genomic DNA was used as a template to amplify a 1.5 kb DNA fragment of the 16S rRNA gene using the universal primer pair 27F and 1492R (Table 2). Polymerase chain reaction (PCR) mixtures and conditions were described in Cherif-Antar et al. (2016). Amplicons were purified using GenElute™ PCR Clean-Up columns (Sigma-Aldrich) and sequenced. Sequences were compared to those deposited in the NCBI database using the BLAST program (https://blast.ncbi.nlm.gov/Blast.cgi), and to those in the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp).

The strains were genetically typed by random amplification of polymorphic DNA-PCR (RAPD-PCR) using primers BoxA2R, M13 and OPA18 (Table 2). The PCR reaction mixtures contained 2 μl each of purified genomic DNA, 12.5 μl Taq DNA Polymerase 2x Master Mix RED

Table 2
Primers used for the identification, PCR fingerprinting and detection of the genes encoding the enzymes glutamate decarboxylase (*gadB*), tyrosine decarboxylase (*tdcA*), histidine decarboxylase (*hdcA*), ornithine decarboxylase (*odc*) and agmatine deiminase (*aguA-aguD*) involved GABA, tyramine, histidine and putrescine biosynthesis.

Name	Target	Sequence	Product size (bp)	Reference
27F 1492R	16S rRNA	5′-AGAGTTTGATCCTGGCTCAG-3′ 5′-GGTTACCTTGTTACGACTT-3′	1465	Cherif-Antar et al. (2016)
BoxA2R	RAPD	5′-ACGTGGTTTGAAGAGATTTTCG-3′		Koeuth et al. (1995)
M13		5′-GAGGGTGGCGGTTCT-3′		Rossetti and Giraffa (2005)
OPA18		5′-AGGTGACCGT-3′		Mättö et al. (2004)
GadL-F GadL-R	*gadB* [a]	5′-TACAATATGCCTTTTCTTTTAG-3′ 5′-AATCACTCATTTCGGTATAC-3′	1401	Valenzuela et al. (2019)
GadP-F GadP-R	*gadB* [b]	3′-ATGGCAATGTTATACGGTAAAC-5′ 3′-GTGTGTGAATCCGTATTTCTTAG-5′	1410	Valenzuela et al. (2019)
GadB-F GadB-R	*gadB* [c]	3′-AGGCAGTGTCGAAGCCGGGCAA-5′ 3′-CATGGATGGGCGTACCACGATCC-5′	1300	Renes et al. (2017)
tdc1 *tdc2*	*tdcA*	5′-TACCGTTACAATATGCCATTTG-3′ 5′-CACACACTTAGGCATAAAGAATC-3′	720	Fernández et al. (2004)
HDC3 HDC4	*hdcA*	5′-GATGGTATTGTTTCKTATGA-3′ 5′-CCAAACACCAGCATCTTC-3′	435	Coton et al. (2010)
ODC1 ODC2	*odc*	5′-NCAYAARCAACAAGYNGG-3′ 5′-GRTANGGNTNNGCACCTTC-3′	900	Coton et al. (2010)
Seq1 Seq2	*aguA-aguD*	5′-CAAGATTTDTTCTGGGCHTTYTTCTC-3′ 5′-TTGGHCCACARTCACGAACCCT-3′	700	Ladero et al. (2011)

[a] Specific primers for *Lc. lactis*.
[b] Specific primers for *L. plantarum*.
[c] Specific primers for *L. brevis*.

(Ampliqon, Odense, Denmark), 5 μl primer (10 μM) and molecular biology grade water (Sigma-Aldrich) in a total volume of 25 μl. The PCR conditions were: one cycle at 95 °C for 7 min, 40 cycles at 95 °C for 30 s, annealing for 1 min at 40 °C for primer BoxA2R, 42 °C for primer M13 or 32 °C for primer OPA18, 72 °C for 4 min and a final extension cycle at 72 °C for 10 min. PCR profiles were visualised on 2.5% agarose gels after electrophoresis at 75 V for 120 min and photographed under UV light. The banding patterns were clustered using the unweighted pair group method with arithmetic mean (UPGMA) and their pattern similarity was expressed by the simple matching (SM) coefficient using GeneTools software v.4.03 (SynGene, Cambridge, UK). To test the reproducibility of the PCR fingerprinting technique, three DNA extractions of the same strain (*Lc. lactis* TAUL227) were performed. Subsequently, amplifications were performed with the three primers used and the lowest similarity percentage was determined. Reproducibility studies showed a similarity percentage of more than 84%. Therefore, profiles with ≥84% similarity were considered to be the same strain.

2.3. Enzymatic activity profile

The API-ZYM system (BioMérieux, Marcy-L'Etoile, France) was used to evaluate enzymatic activities of potential interest in LAB (enzymes listed in Table 3). Appropriate liquid media were inoculated with each LAB strain and incubated for 16 h at 32 °C. Cultures were centrifuged at 7000×*g* for 15 min at 4 °C. The supernatants were discarded and the bacterial cells were resuspended in 2 ml phosphate buffer (50 mM, pH 7.0) until they reached an optical density equivalent to McFarland 5. The cell suspensions were then inoculated into the microtubes of the API-ZYM strip and incubated according to the manufacturer's instructions. Enzyme activity was graded from 0 to 5 by comparing the colour developed within 5 min with the API-ZYM colour reaction chart (Durlu-Ozkaya et al., 2001). The results were expressed in nmol of substrate hydrolysed from the intensity of the reactions obtained, ranging from 0 (no activity) to 5 (40 or more nanomoles released).

2.4. Glutamate dehydrogenase (GDH) assay

GDH activity was assayed in cell-free extracts (CFE) of each of the LAB strains obtained by mechanical disruption in a MiniBead Beatter (Biospec, Oklahoma, USA) according to the method described by Abarquero et al. (2022). The GDH activity of CFE was determined by the Boehringer glutamic acid colourimetric assay using a commercial glutamic acid colourimetric assay (R-Biopharma, Germany) as described in Lee et al. (2020). The reaction mixture contained 80 μL distilled water, 80 μL potassium phosphate/triethanolamine buffer (pH 8.6), 40 μL 100 mM L-glutamic acid (Sigma-Aldrich), 40 μL iodonitrotetrazolium and 40 μL NAD-diaphorase (600 μL in total). After adding 60 μl of CFE to the reaction mixture, 200 μl aliquots of the mixture were immediately added to each of three wells and incubated for 1 h at 30 °C and the absorbance measured at 492 nm. To subtract non-specific reactions that could produce the reduced cofactors, a control assay was prepared for each strain without the addition of L-glutamic acid. GDH activity was expressed as the increase in absorbance at 492 nm per mg CFE protein per minute of reaction.

2.5. Detection of antimicrobial activity

An agar-well diffusion assay was used to determine the antibacterial activity of LAB strains against six reference strains of pathogenic or spoilage character (listed in Table 1). One milliliter of a cell suspension (McFarland's standard 0.5) obtained from an overnight culture of each reference strain was inoculated into 15 ml of appropriate semi-solid agar (broth plus 0.7% bacteriological agar). After solidification, 10 mm diameter wells were cut and 35 μl of overnight cultures of the test strains were added. Plates were incubated for 24 h at the appropriate temperature for each reference strain and checked for zones of inhibition. To determine whether the inhibition was due to acidity, a new agar well diffusion test was performed after neutralization of the supernatants at pH 6.0. Finally, to verify that the inhibition was due to the production of bacteriocin-like compounds and not to the production of hydrogen peroxide, the neutralised supernatants (pH 6.0) were treated with a sterile catalase solution (1 mg ml^{-1}).

Table 3
Enzymatic activity[a] (approximate values), detected using API-ZYM system, of whole cells and specific glutamate dehydrogenase activity of 20 wild lactic acid bacteria strains.

Strain	Enzymes tested[b]																		GDH activity
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	(U $mg^{-1}min^{-1}$)[c]
Lactococcus lactis																				
GE44	5	10	10	5	≥40	10	10	0	5	30	10–20	0	0	0	5	0	0	0	0	–[4]
TAUL88	0	5	5–10	<5	≥40	10	20	0	0	≥40	5	0	<5	0	5	0	0	0	0	–
TAUL227	5	5	5	0	≥40	5	10	0	0	≥40	10	0	0	0	0	0	0	0	0	0.018 ± 0.004 [abc]
TAUL266	5	5	5	0	30	5	10	0	0	30	20	0	0	0	0	0	0	0	0	–
TAUL8000	0	5	5–10	0	≥40	5	10–20	0	0	≥40	30–40	0	<5	0	5	0	0	0	0	0.008 ± 0.000 [abc]
TAUL9000	0	5	<5	0	30	<5	10	0	0	≥40	10	0	0	0	0	0	0	0	0	0.015 ± 0.002 [abc]
Leuconostoc mesenteroides																				
TAUL1342	0	5	0	0	5	0	5	0	0	5	5	5	≥40	0	30	≥40	0	0	0	0.009 ± 0.001 [abc]
Lactiplantibacillus paraplantarum																				
TAUL1365	10	10	5	5	≥40	5	10	0	0	10–20	10–20	5	≥40	0	0	5–10	10–20	0	0	0.001 ± 0.000 [a]
TAUL1399	0	5	0	0	≥40	20	10	0	0	10	5	0	≥40	0	5	20	≥40	0	0	0.003 ± 0.001 [abc]
Lactiplantibacillus plantarum																				
TAUL67	5	<5	<5	0	≥40	30	20–30	0	0	20–30	10–20	20	≥40	0	30	30–40	≥40	0	0	0.001 ± 0.000 [abc]
TAUL188	0	0	0	0	≥40	30–40	20	0	0	5–10	10–20	0	≥40	0	30	30–40	30–40	0	0	–
TAUL1368	<5	0	<5	0	≥40	30	20	0	0	30	10–20	10	≥40	0	≥40	30–40	≥40	0	0	–
TAUL1641	0	0	0	0	≥40	30	20	0	0	10	10–20	0	≥40	0	30–40	≥40	≥40	0	0	0.098 ± 0.012 [c]
TAUL1667	5	5	5	0	≥40	30	30	0	0	30	≥40	0	≥40	5	≥40	20	≥40	0	0	0.009 ± 0.003 [abc]
TAUL1692	0	0	0	0	≥40	30	20	0	0	20	10	0	≥40	0	0	30	≥40	0	0	–
TAUL1700	0	5	5	5	30	30	30	0	0	10–20	10–20	0	≥40	0	≥40	10–20	5	0	0	0.008 ± 0.005 [abc]
TAUL1765	0	5	5	5	≥40	30	20	0	0	20	20	0	≥40	0	≥40	20	≥40	0	0	0.009 ± 0.003 [abc]
Levilactobacillus brevis																				
TAUL1567	0	5	0	0	≥40	30	5	0	0	≥40	≥40	0	≥40	30	30	≥40	0	0	0	0.049 ± 0.001 [bc]
Lacticaseibacillus paracasei																				
TAUL1508	0	10	10	0	≥40	≥40	5	0	5	20	20–30	0	≥40	0	30–40	0	0	0	0	0.001 ± 0.000 [ab]
TAUL1752	0	5	5	0	≥40	30	5	0	0	20	30	0	≥40	0	≥40	30	5	0	0	–

[a] Enzymatic activity (approximate values) expressed as nmol of substrate hydrolysed.

[b] Enzymes tested: 1 – Alkaline phosphatase; 2 – Esterase (C4); 3 – Esterase lipase (C8); 4 – Lipase (C14); 5 – Leucine arylamidase; 6 – Valine arylamidase; 7 – Cystine arylamidase; 8 – Trypsin; 9 – α-Chymotrypsin; 10 – Acid phosphatase; 11 – Naphthol-AS-BI-phosphohydrolase; 12 – α-galactosidase; 13 – β-galactosidase; 14 – β-glucuronidase; 15 – α-glucosidase; 16 - β-glucosidase; 17 - N-acetyl-β-glucosaminidase; 18 – α-manosidase; 19 – α-fucosidasa.

[c] Specific GHD activity expressed as increase in A_{492} per milligram of protein of CFE and per minute of reaction. [4]not detected. [abc] Values for different strains that do not show a common superscript differ significantly ($p < 0.05$).

2.6. Antibiotic susceptibility

Minimum inhibitory concentrations (MICs) of 16 antibiotics (listed in Table 4) were determined by microdilution using Sensititre EULACBI1 and EULACBI2 plates (Trek Diagnostic Systems, East Grinstead, UK) according to EFSA guidance on the use of production microorganisms (EFSA FEEDAP Panel, 2018). Individual colonies were suspended in sterile saline (0.9% NaCl) until a density equivalent to McFarland standard 1 was reached. The suspension was then diluted 1000-fold in IsoSensitest (IST) broth (Oxoid) (for *Lactococcus*) or LSM medium (90% IST + 10% MRS) (for *Lactobacillus* and *Leuconostoc*). A 100 μl aliquot of this suspension was added to each well of the Sensititre plates and incubated under aerobic conditions at 32 °C for 48 h. MICs were defined as the lowest concentration (μg ml^{-1}) at which no visible growth was observed.

2.7. Quantification of tyramine, histamine, putrescine and GABA production by UHPLC

The production of tyramine, histamine and putrescine in liquid cultures was assayed in appropriate media supplemented with 1 mM of their respective precursor amino acids (tyrosine, histidine, agmatine or ornithine; all from Sigma-Aldrich, St. Louis, MO., USA), while for the quantification of GABA production, appropriate liquid media were supplemented with 5 mM monosodium glutamate (MSG, Sigma-Aldrich) (Valenzuela et al., 2019). The supplemented media were inoculated with each LAB strain and incubated for 48 h under optimal conditions.

After incubation, the cultures were centrifuged (15,000×*g* for 10 min). The supernatants were filtered through a 0.45 μm cellulose membrane (Millipore, Burlington, MA, USA). Aliquots of 100 μl were derivatised with diethyl ethoxymethylene malonate (DEEMM; Sigma-Aldrich) as previously described (Valenzuela et al., 2019). L-2-amino-adipic acid (Sigma-Aldrich) was used as an internal standard. Ten μl of the derivatised sample was subjected to ultra-high performance liquid chromatography (UHPLC) using a Waters H-Class ACQUITY UPLC system coupled to a photodiode array detector (Waters, Milford, MA, USA) and separation was performed using a Waters Acquity UPLC™ BEH C18 column (1.7 μm particle size, 100 mm × 2.1 mm I.D.) according to the method described by Redruello et al. (2013).

2.8. Detection of GABA and biogenic amines production genes

The presence of the glutamate decarboxylase (*gadB*), tyrosine decarboxylase (*tdcA*), histidine decarboxylase (*hdcA*), ornithine decarboxylase (*odc*) genes and agmatine deiminase cluster (*aguA-aguD*) was determined by PCR amplification using the specific primers described in Table 2. The PCR reaction mixtures contained 1 μl of each purified genomic DNA, 12.5 μl of Taq DNA Polymerase 2x Master Mix RED, 1 μl of each primer (10 μM), and molecular biology grade water in a total volume of 25 μl. The PCR conditions were One cycle at 95 °C for 5 min, 35 cycles at 95 °C for 45 s, annealing for 1 min at 56 °C for GadL-F/GadL-R, GadP-F/GadP-R and GadB-F/GadB-R, 1 min at 50 °C for primers tdc1/tdc2, 1 min at 52 °C for primers HDC3/HDC4 and ODC1/ODC2 or 25 s at 52 °C for primers Seq1/Seq2; and 72 °C for 2 min; and a final extension cycle at 72 °C for 10 min. Amplification was visualised on 2.5% agarose gels after electrophoresis at 75 V for 60 min. Amplicons were purified

Table 4
Minimum inhibitory concentration (MIC) to 16 antibiotics of 20 wild lactic acid bacteria strains.

Strain	Antibiotic[a] (MIC as μg ml^{-1})															
	GEN	KAN	STR	NEO	TET	ERY	CLI	CHL	AMP	PEN	VAN	SYN	LZD	TMP	CIP	RIF
Lactococcus lactis																
GE44	1	4	8	2	0.12	0.03	≤0.03	1	0.06	0.06	≤0.25	0.5	1	≥64	2	2
TAUL88	1	8	16	8	0.12	0.03	0.03	1	0.12	0.12	0.25	0.5	0.5	≥64	2	8
TAUL227	0.5	2	8	1	0.25	0.015	≤0.03	1	0.06	0.12	≤0.25	1	0.5	≥64	2	8
TAUL266	≤0.5	2	4	1	0.25	0.03	≤0.03	1	0.06	0.12	≤0.25	0.5	0.5	≥64	2	8
TAUL8000	1	8	16	4	0.12	0.03	≤0.03	1	0.12	0.12	0.25	0.5	0.5	≥64	2	8
TAUL9000	1	4	8	1	0.5	0.03	0.06	2	0.12	0.25	0.25	2	1	≥64	2	8
Cut-off[b]	**32**	**64**	**32**	-[c]	**4**	**1**	**1**	**8**	**2**	-	**4**	-	-	-	–	-
Leuconostoc mesenteroides																
TAUL1342	≤0.5	4	4	0.25	1	0.03	≤0.03	2	0.5	0.25	≥128	0.25	1	4	2	0.25
Cut-off	**16**	**16**	**64**	-	**8**	**1**	**1**	**4**	**2**	-	**i.r.**[4]	-	-	-	–	-
Lactiplantibacillus paraplantarum																
TAUL1365	≤0.5	≤2	1	≤0.12	4	0.015	0.03	0.5	0.06	0.25	≥128	0.25	0.5	≤0.12	0.25	0.12
TAUL1399	≤0.5	≤2	4	0.25	4	0.03	0.25	1	2	16	≥128	0.25	0.5	≤0.12	4	0.25
Lactiplantibacillus plantarum																
TAUL67	≤0.5	8	8	0.5	8	0.03	0.12	2	1	4	≥128	0.5	2	1	8	0.5
TAUL188	≤0.5	8	8	0.5	8	0.03	0.12	2	1	16	≥128	0.5	2	≤0.12	8	1
TAUL1368	≤0.5	4	4	0.25	8	0.06	0.06	2	1	4	≥128	0.5	1	≤0.12	8	0.5
TAUL1641	≤0.5	4	4	0.25	8	0.03	0.03	2	1	8	≥128	0.5	1	0.12	16	1
TAUL1667	≤0.5	4	4	0.5	16	0.03	0.06	2	1	16	≥128	1	1	0.12	8	0.5
TAUL1692	≤0.5	8	4	0.25	4	0.03	0.03	1	0.06	0.25	≥128	0.25	0.5	≤0.12	4	0.25
TAUL1700	≤0.5	8	4	0.25	2	0.06	0.03	1	1	0.25	≥128	0.25	0.5	≤0.12	4	0.25
TAUL1765	≤0.5	4	4	0.25	2	0.03	0.03	1	0.06	0.25	≥128	0.25	0.5	≤0.12	4	0.25
Cut-off	**16**	**64**	–	-	**32**	**1**	**4**	**8**	**2**	-	**i.r.**	-	-	-	-	-
Levilactobacillus brevis																
TAUL1567	≤0.5	2	2	≤0.12	8	0.015	0.25	2	0.25	0.5	≥128	0.25	1	≤0.12	4	0.12
Cut-off	**16**	**16**	**64**	-	**8**	**1**	**4**	**4**	**2**	-	**i.r.**	-	-	-	–	-
Lacticaseibacillus paracasei																
TAUL1508	2	16	16	4	1	0.03	0.03	2	0.5	0.25	≥128	0.25	0.5	2	1	≤0.12
TAUL1752	1	16	16	2	0.5	0.015	≤0.03	2	0.5	0.25	≥128	0.25	1	0.5	1	0.12
Cut-off	**32**	**64**	**64**	-	**4**	**1**	**4**	**4**	**4**	-	**i.r.**	-	-	-	–	-

[a] GEN, gentamicin; KAN, kanamycin; STR, streptomycin; NEO, neomycin; TET, tetracycline; ERY, erythromycin; CLI, clindamycin; CHL, chloramphenicol; AMP, ampicillin; PEN, penicillin; VAN, vancomycin; SYN, quinupristin-dalfopristin; LZD, linezolid; TMP, trimethoprim; CIP, ciprofloxacin; RIF, rifampicin.
[b] Cut-off expressed in μg ml^{-1}.
[c] -, cut-off not established. [4] i. r., intrinsically resistant.

using GenElute PCR Clean-Up columns and sequenced. The sequences obtained were assembled and compared with those in databases using BLAST software (https://blast.ncbi.nlm.gov/Blast.cgi). DNA sequences were aligned using the Clustal W algorithm included in the MEGA11 software and their phylogenetic relationships calculated using the Maximum Likelihood-Ratio Test (Tamura et al., 2021). The *gadB* gene sequences from *Lc. lactis* NCDO2727 (GenBank Accession no. MK225577.1) (Laroute et al., 2022), *L. plantarum* GM1403 (GenBank accession no. MN991271.1) (Lyu et al., 2021) and *L. brevis* NCL912 (GenBank accession no. JX074764.2) (Li et al., 2013) were used as comparators.

2.9. Statistical analysis

Statistical analysis of GABA production and GDH activity were performed using SPSS v.25 (SPSS, Chicago, IL, USA). Due to the non-normal distribution of the data, the Kruskal-Wallis test was used to determine statistical differences ($p < 0.05$) between strains. To analyse differences between groups, a post-hoc Mann-Whitney test was used and significant results were adjusted using the Bonferroni correction.

3. Results and discussion

3.1. Molecular identification and PCR fingerprinting

The ascription of the strains to the species level, previously identified by Maldi TOF/MS (Table 1), was confirmed by 16S rRNA gene sequencing and the sequences were compared with databases.

Using a threshold of 84% identity obtained in the reproducibility study, 20 different RAPD profiles were independently obtained with BoxA2R, M13 and OPA18 primers. Therefore, all these 20 profiles were considered to belong to different strains: *Lc. lactis* (6), *Ln. mesenteroides* (1), *L. paraplantarum* (2), *L. plantarum* (8), *L. brevis* (1) and *L. paracasei* (2). This typing revealed that *L. paraplantarum* isolates TAUL1365 and TAUL1453 were probably the same strain. Also, *L. plantarum* isolates TAUL67 and TAUL180, as well as isolates TAUL1569 and TAUL1641, which were considered to be the same strain, and isolates TAUL1692 and TAUL1694, which also had the same RAPD profile.

3.2. Enzymatic activity profile

The enzyme activities of the 20 LAB strains evaluated using the API-ZYM system are shown in Table 3. No or very low alkaline phosphatase activity was detected in the strains tested, which seems to be common among LAB. None of the strains showed activity (or very low activity) for trypsin, α-manosidase and α-fucosidase. β-glucuronidase, a rare activity in LAB, was only detected in *L. brevis*. This enzyme is associated with the production of compounds of a toxic and carcinogenic nature, making it an undesirable activity among starter culture strains (Michlmayr & Kneifel, 2014). In contrast, high levels of N-acetyl-β-glucosaminidase were found in *L. paraplantarum* and *L. plantarum* strains. These results are in agreement with those reported by Karakas-Sen and Karakas (2018) for some *L. plantarum* strains isolated from raw milk. Esterase (C4) and esterase-lipase (C8) activities were low or undetectable (from 0 to 10 nmol of substrate hydrolysed), while lipase (C14) activity was detected only in two strains of *L. plantarum*, one strain of *L. paraplantarum* and two strains of *Lc. lactis* (5 nmol of substrate hydrolysed). However, even low levels of lipolytic activity in cheese starter cultures may be important for flavour development in cheeses, especially in long-ripened cheeses, due to the low detection threshold of compounds resulting from lipolytic activity (Domingos-Lopes et al., 2017). Acid phosphatase activity, an essential enzyme for the hydrolysis of phosphopeptides prevalent in cheese ripening (Domingos-Lopes et al., 2017), showed values between 5 and more than 40 nmol of hydrolysed substrate, being particularly high in *Lc. lactis* strains. On the other hand, the proteolytic activities of LAB generate peptides and free amino acids that act as precursors in decarboxylation, deamination, transamination and desulphurisation reactions, which play a crucial role in determining the flavour of foods (García-Cano et al., 2019). Leu-arylamidase activity was detected in all strains, with significant activity (in the order of 40 nmol of hydrolysed substrate) in most strains. Val-arylamidase activity was high in lactobacilli strains, while Cys-arylamidase activity was somewhat lower but was also detected in Lactococcus strains. The presence of strong Leu-arylamidase activity detected in most LAB strains is desirable for use as a starter, as aminopeptidases release amino acids during cheese ripening, which can be metabolised by bacterial enzymes into other products, playing an interesting role in cheese flavour (Câmara et al., 2019; Domingos-Lopes et al., 2017). It can also reduce bitterness by hydrolysing bitter peptides formed in cheese (González et al., 2015). Most strains of the lactobacilli and *Leuconostoc* showed high β-galactosidase, α-glucosidase and β-glucosidase activity (30–40 nmol hydrolysed substrate), while no or very low activity was detected in *Lc. lactis* strains. These results are consistent with those reported by other authors (Câmara et al., 2019; Mechai et al., 2014). β-Galactosidase is involved in the acidification process of milk, may contribute to the alleviation of lactose intolerance, and its activity leads to the formation of galactooligosaccharides, which have a prebiotic and bifidogenic effect, so the selection of LAB with high β-galactosidase activity may be interesting from both a technological and probiotic point of view (Câmara et al., 2019). β-glucosidase is important because, in combination with other enzymes, it contributes to the hydrolysis of polysaccharides such as cellulose, and α-glucosidase can hydrolyse maltose and are therefore interesting properties for probiotic LAB strains (Michlmayr & Kneifel, 2014).

3.3. Glutamate dehydrogenase activity

The results of the GDH activity assay (Table 3) showed that 13 of the 20 LAB strains included in this study had GDH activity. However, the activity values were significantly different ($p < 0.05$), with *L. plantarum* TAUL1641 and *L. brevis* TAUL1567 strains having the highest activity values (0.098 and 0.049 U mg-1 min-1, respectively). These results confirm that although GDH activity is a ubiquitous enzyme, its presence is highly species and strain dependent (Tanous et al., 2002). In comparison with other assays, our results are similar to those obtained by Kieronczyk et al. (2003) or Lee et al. (2020) who also tested starter and non-starter LAB strains. In these works, GDH activity was also found to be strain dependent, with activity values ranging from 0.001 to 0.2 U mg^{-1} min^{-1}.

Cheese flavours are produced by a complex process and are the result of a precise balance and concentration of a variety of volatile compounds, many of which are directly derived from amino acid catabolism (Smit et al., 2005). GDH activity is crucial in this catabolic flux, as transamination is dependent on the availability of α-ketoglutarate. Therefore, selection of LAB strains on the basis of this activity can be very beneficial for strain inclusion in new cheese starter cultures (Lee et al., 2020; Mazhar et al., 2020).

3.4. Antimicrobial activity

The agar-well diffusion assays against the six reference microorganisms (Table S1) showed that antimicrobial effect of the strains disappeared when the supernatants were neutralised (pH 6.0), except for *Lc. lactis* GE44 that inhibited *L. monocytogenes* CTCE4031 and *L. plantarum* TAUL1667 that inhibited *E. faecalis* CTCE481. However, when the neutralised supernatants were treated with catalase, only *L. plantarum* TAUL1667 inhibited the growth of *E. faecalis* CTCE481. In view of the results, most of the strains inhibited the growth of the reference strains by lowering the pH of the medium, resulting from the production of organic acids. In addition, *Lc. lactis* strain GE44 inhibited the growth of *L. monocytogenes* CTCE4031 by H_2O_2 production; while *L. plantarum* TAUL1667 inhibited the growth of *E. faecalis* CTCE481 by

another type of antimicrobial compound, possibly a bacteriocin-like compound.

In recent years, bacteriocins have attracted much interest as potential antimicrobials for food preservatives (Tang et al., 2022). Traditional fermented dairy products represent an important niche for LAB isolates capable of inhibiting the growth of spoilage indicator and pathogenic bacteria (Agostini et al., 2018). However, the production of inhibitory compounds in LAB, such as bacteriocins, is a strain-specific trait, so finding producing strains requires screening large numbers of strains. In previous studies, the proportion of LAB strains producing antimicrobial compounds has been variable, ranging from studies detecting a high proportion of strains producing bacteriocin-like compounds (Marchwińska & Gwiazdowska, 2022; Tulini et al., 2016) to others where the proportion was much lower (Agostini et al., 2018; Câmara et al., 2019). Therefore, it is not surprising that in a small group of strains, such as those included in this study, only one strain was found to be able to inhibit the growth of one of the indicator microorganisms.

3.5. Antibiotic susceptibility

The presence of transferable antibiotic resistance is a key criterion for the selection of strains for use in feed and food (EFSA FEEDAP Panel, 2018; Sirichoat et al., 2020). Table 4 shows the results for antibiotic susceptibility against a set of 16 antibiotics using a broth microdilution method. Focusing on lactobacilli, they are generally sensitive to tetracycline, erythromycin, chloramphenicol, penicillin, ampicillin, clindamycin, quinupristin-dalfopristin, linezolid and rifampicin (Anisimova & Yarullina, 2019; Sirichoat et al., 2020), while they are intrinsically resistant to vancomycin and ciprofloxacin. Reference values for most LAB species beyond those provided by EFSA are lacking. The resistance of *Lc. lactis* strains to high levels of trimethoprim has been repeatedly reported and is considered intrinsic (Huys et al., 2002; Katla et al., 2001). In the present study, two strains had MICs equal to the limits established to separate susceptible from resistant strains (EFSA FEEDAP Panel, 2018): *L. paraplantarum* TAUL1399, which had a MIC of 2 μg ml^{-1} against ampicillin, and *L. brevis* TAUL1567, which had a MIC of 8 μg ml^{-1} against tetracycline. MICs equal to or one dilution higher than the cut-offs are considered to be within the normal variation of the microdilution assay and not of concern (Huys et al., 2010). Nevertheless, it may be appropriate to screen for genes involved in resistance to these antimicrobials before using them as starters.

3.6. Biogenic amines production and detection of their production genes

The detection of strains producing toxic biogenic amines is another criterion of interest when including or not including LAB strains in starter cultures. In fermented dairy products, especially cheese, proteolysis leads to the release of free amino acids that can be converted into toxic BAs (Tofalo et al., 2019). The production and accumulation of toxic BAs in cheese, such as histamine and tyramine, can be detrimental to health (Tittarelli et al., 2019). Table 5 shows the LAB producing strains and the concentrations of BAs detected by UHPLC and the presence/absence of the *tdcA*, *hdcA* and *odc* genes and the *aguA-aguD* gene. There was no production of any of the BAs studied and no amplification of any of the genes studied in any of the *L. paraplantarum*, *L. paracasei* and *Ln. mesenteroides* strains. No histamine or putrescine (from ornithine) production or amplification of the *hdcA* and *odc* genes was detected in any of the *Lc. lactis*, *L. plantarum* and *L. brevis* strains. Tyramine production was only detected in *L. brevis* TAUL1567 strain, at a concentration of 193.15 μg ml^{-1} and PCR detection of the *tdc* gene was positive. However, *tdc* gene amplifications were also obtained in *Lc. lactis* TAUL88 and TAUL227 but tyramine production was not detected under the conditions studied. *Lc. lactis* is the most widely used LAB species as a starter culture for the production of cheese and other fermented dairy products. It should be noted that, although some strains have been identified as tyramine producing, tyramine production by *Lc. lactis* is not common. Putrescine production from agmatine was detected in *Lc. lactis* TAUL88 and TAUL8000 strains and in *L. brevis* TAUL1567, which showed the highest concentration (52.27 μg ml^{-1}). Two types of results were obtained for the *aguA-aguD* gene clusters, two types of results were obtained: 700 bp amplicons in *L. brevis* TAUL1567 and *Lc. lactis* TAUL88, TAUL8000 and TAUL9000; and 1500 bp amplicons for *Lc. lactis* GE44, TAUL227 and TAUL266.

As can be seen, in some of the strains the presence of the genes involved was detected by PCR, but the production of BAs was not. This could be due to an alteration of the genes or other genes involved in the synthesis. In the case of *Lc. lactis* GE44, TAUL227 and TAUL266, the sequence of the cluster *aguA-aguD* was 1500 bp in size. When compared with the sequences deposited in the databases, 98.34% identity was obtained with the sequence of the *aguD* gene disrupted by the IS*983N* transposase (GenBank accession no. FR856582.1). As reported in the work of Ladero et al. (2011), the presence of this IS element disrupts the transcription of the AGDI cluster, preventing the production of

Table 5
GABA, tyramine and putrescine production (μg ml^{-1}) in 15 out of 20 wild lactic acid bacteria strains[a] and detection *gadB, tdcA* genes and *aguA-aguD gene* cluster by polymerase chain reaction amplification.

Species	Strain	GABA[b]	*gadB*	Tyramine[b]	*tdcA*	Putrescine[b]	*aguA-aguD*
Lactococcus lactis	GE44	211.29 ± 3.01	+	–	–	–	IS[c]
	TAUL88	58.47 ± 4.54	+	–	+	43.11 ± 0.22	+
	TAUL227	62.80 ± 1.75	+	–	+	–	IS
	TAUL266	69.92 ± 0.03	+	–	–	–	IS
	TAUL8000	54.76 ± 0.08	+	–	–	43.81 ± 0.64	+
	TAUL9000	-[d]	+	–	–	–	+
Lactiplantibacillus plantarum	TAUL67	330.40 ± 0.42	+	–	–	–	–
	TAUL188	115.29 ± 1.65	+	–	–	–	–
	TAUL1368	79.61 ± 1.28	+	–	–	–	–
	TAUL1641	91.16 ± 2.04	+	–	–	–	–
	TAUL1667	54.34 ± 1.09	+	–	–	–	–
	TAUL1692	4.23 ± 0.01	+	–	–	–	–
	TAUL1700	177.78 ± 0.91	+	–	–	–	–
	TAUL1765	98.07 ± 0.33	+	–	–	–	–
Levilactobacillus brevis	TAUL 1567	845.99 ± 8.11	+	193.15 ± 0.09	+	52.27 ± 0.28	+

[a] Lactic acid bacteria strains in which the production of the compound and/or the presence of the genes involved have been detected. In the remaining five strains, neither the compound nor the genes involved were detected.
[b] GABA, tyramine and putrescine production in liquid cultures supplemented with 5 mM monosodium glutamate, 1 mM tyrosine or 1 mM agmatine, respectively; quantified by UHPLC analysis after derivatization. Results expressed as mean values ± standard deviation of two biologically-independent replicates.
[c] IS = Positive amplification of the *aguA-aguD* fragment including the transposase IS*983N* sequence (GenBank accession no. FR856582.1).
[d] (–) not detected.

putrescine from agmatine. On the other hand, although the *tdcA* gene was detected in *Lc. lactis* strains TAUL88 and TAUL227, no production of this BA was recorded; therefore, it would be useful to evaluate the production of tyramine in these strains under different culture conditions.

3.7. GABA production and detection of glutamate decarboxylase gene

GABA production was detected in the eight *L. plantarum* strains, in five *Lc. lactis* strains and in the *L. brevis* strain, which had the highest production with a GABA concentration of 845.99 μg ml^{-1} (Table 5). In concordance with these results, other authors have reported GABA production by LAB isolated from traditional cheeses, including strains of different species (*L. plantarum*, *L. brevis* and *Lc. lactis*) (Cui et al., 2020; Redruello et al., 2021). However, GABA production differed from that found in other studies (Jitpakdee et al., 2021; Kanklai et al., 2021). In the latter, the much higher production may be due to MSG acting as a limiting factor for production. Thus, the incorporation of a low concentration of MSG into the culture could be the reason for the lower GABA production.

As several GABA-producing strains of *L. plantarum* and *Lc. lactis* were found, the sequence of the *gadB* gene in these strains was compared to see if there were differences between them. Positive amplifications of the *gadB* gene were obtained in all GABA-producing strains (Table 5). The internal segments covering most of the amplified GAD genes were sequenced, and the sequences obtained showed very high percentages of identity with the *gadB* gene sequences of *Lc. lactis* (>98%), *L. brevis* (>98%) and *L. plantarum* (>97%). Fig. 1 shows a phylogenetic tree of the internal segment of the *gadB* genes from *Lc. Lactis*, *L. plantarum* and *L. brevis* strains. The multiple sequence alignment showed that the GAD sequences of the six *Lc. lactis* strains were almost identical. Only one nucleotide substitution was found in the sequences of *Lc. lactis* strains TAUL88 and TAUL8000 compared to all other strains. In contrast, a total of 34 nucleotide substitutions were found in the sequences of the *L. plantarum* strains. As shown in Fig. 1, two types of sequences can be distinguished phylogenetically in this species: a first group consisting of *L. plantarum* strains TAUL188, TAUL1692, TAUL1700, TAUL1765 (more similar to the *L. plantarum* GM1403 sequence); and a second group consisting of *L. plantarum* strains TAUL67, TAUL1368, TAUL1667 and TAUL1641. Despite this sequence heterogeneity, no relationship was found between the two groups and GABA production in *L. plantarum* strains. The explanation for this may be that the polymorphisms do not affect the active site of the enzyme or the binding site of pyridoxal-5′-phosphate, a cofactor of the decarboxylation reaction (Lyu et al., 2021). However, variations in the amount of GABA produced by the strains included in the study, both *Lc. lactis* and *L. plantarum*, may be due to the genetic background involved in the GABA biosynthesis pathway (Yogeswara et al., 2020).

4. Conclusions

The selection of LAB strains through technological characterization is a key element in the search for new strains for the development of starter cultures for cheese, but before testing the cultures in cheese production, it is essential to study the safety of the strains to avoid that the selected strains pose a health risk. In this respect, the study of the different enzymatic activities showed high values of proteolytic activity, especially in *L. plantarum strains*, while the values of lipolytic activities were higher in *Lc. lactis* strains; and the specific study of GDH activity in LAB strains showed that this key activity in amino acid catabolism was present in 13 strains, with *L. plantarum* TAUL1641 and *L. brevis* TAUL1567 strains showing the highest values. On the other hand, *L. plantarum* TAUL1667 showed antimicrobial activity against *E. faecalis* after neutralization and catalase treatment of the supernatant; and one strain of *Lc. lactis*, three strains of *L. plantarum* and one strain of *L. brevis* produced GABA above 100 μg ml^{-1}. Finally, the antibiotic resistance study showed that the 20 selected LAB strains are suitable for inclusion in starter cultures. However, three biogenic amine-producing strains were found, which should not be included in starter cultures: *L. brevis* TAUL1567, which produced tyramine and putrescine, and *Lc. lactis*

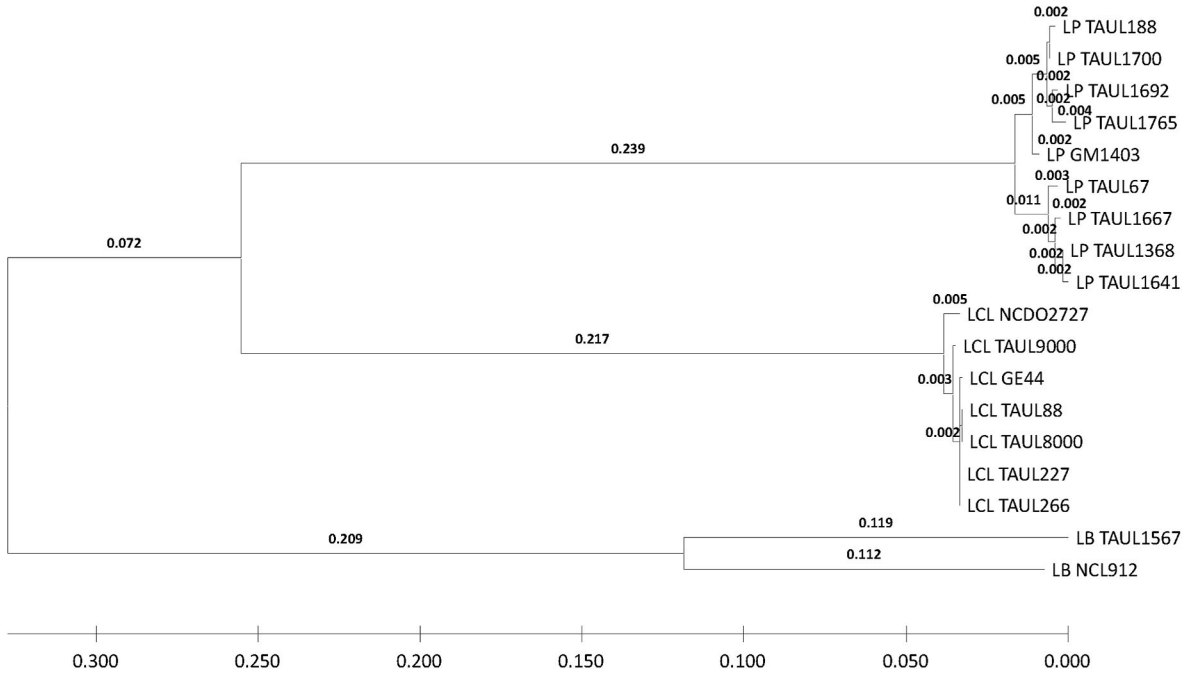

Fig. 1. Phylogenetic tree (maximum-likelihood method) of the internal segment (1,158 bp long) of the different glutamate decarboxylase (*gadB*) genes from the *Lactococcus lactis* (LCL), *Lactiplantibacillus plantarum* (LP) and *Levilactobacillus brevis* (LB) strains. The sequences of *Lactococcus lactis* NCDO2727 (GenBank Accession no. MK225577.1), *Lactiplantibacillus plantarum* GM1403 (GenBank accession no. MN991271.1) and *Levilactobacillus brevis* NCL912 (GenBank accession no. JX074764.2) were used as comparators. Figure was generated from MEGA (v.11) after ClustalW alignment of GAD internal segments. The length of branches is proportional to the number of substitutions per site.

TAUL88 and *Lc. lactis* TAUL8000, which produced putrescine. Based on this study, *Lc. lactis* strain GE44 could be a good candidate for starter culture design, and testing in cheese production; while *L. plantarum* strains TAUL67, TAUL1641 and TAUL1667 could be selected as adjunct cultures based on their enzymatic and antimicrobial activity, respectively.

CRediT authorship contribution statement

Daniel Abarquero: Methodology, Validation, Formal analysis, Investigation, Writing – original draft. **Raquel Bodelón:** Investigation. **Ana Belén Flórez:** Conceptualization, Methodology, Validation. **José María Fresno:** Writing – review & editing, Supervision. **Erica Renes:** Conceptualization, Writing – review & editing. **Baltasar Mayo:** Conceptualization, Writing – review & editing. **María Eugenia Tornadijo:** Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2023.114709.

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